

SYMBIOSIS OF HYDRA AND ALGAE. I. EFFECTS OF SOME ENVIRONMENTAL CATIONS ON GROWTH OF SYMBIOTIC AND APOSYMBIOTIC HYDRA

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Unicellular algae inhabit a variety of aquatic invertebrates. Their primary function as symbionts is in most cases not clearly understood (see review of Droop, 1963). Interest in this problem has led us to an experimental analysis of the association of algae and hydra. Green hydra are particularly suited to an investigation of symbiosis involving a metazoan because (1) methods for mass culture of these hydroids provide a practically unlimited supply of animals of similar genetic, developmental, and nutritional histories, cultured in a fluid of known ionic composition. (2) Aposymbiotic (= algae-free) control hydra are easily obtained (Whitney, 1907) and cultured, and (3) the specific growth rate constant, k (cf. Loomis, 1954), provides a quantitative measure of the effect of various factors, such as ionic composition of the medium, or the presence of symbiotic algae, on growth of the host.

In preparation for studies on the effect of symbiotic algae on growth of hydra, it was necessary first to attempt to control environmental variables affecting growth. Since the most critical of these for laboratory-grown hydroids appears to be the ionic composition of the culture medium (Loomis, 1954; Loomis and Lenhoff, 1956; Ham, Fitzgerald and Eakin, 1956; Lenhoff and Bovaird, 1959, 1960; Fulton, 1962), the experiments described here were undertaken to determine the effect of some environmental cations on growth of symbiotic and aposymbiotic hydra.

MATERIALS AND METHODS

Chlorohydra viridissima, obtained from the Carolina Biological Supply Co., Burlington, N. C., and designated by us "Carolina Strain 1960," was used in all experiments.

The general morphology of green hydra and associated algae is described by Goetsch (1924), Haffner (1925), and Brien and Reniers-Decoen (1950). Present knowledge of symbiotic algae is summarized by Droop (1963).

Carolina Strain 1960, with an average resting length of about 5 mm., is small compared to other green hydra. Usually 15–25 unicellular green algae, each 3–6 microns in diameter, are situated basally within most of the host's gastrodermal cells. Electron micrographs of *C. viridissima* (Wood, 1959) show the intracellular location of the algae.

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The culture medium ("M" solution) for this species of hydra consisted of 10^{-3} M CaCl_2 , 10^{-3} M NaHCO_3 , 10^{-4} M MgCl_2 , 10^{-4} M KCl , and 10^{-3} M tris-(hydroxymethyl)-aminomethane buffer (Sigma "121"), pH 7.6, in water deionized by passing distilled water through organic removal and ion exchange resin columns (Barnstead Black and Red Cap Cartridges). The experimental rationale for the selection of this medium is given in the Results section (Fig. 2). Penicillin G (sodium, U.S.P., 50 mg./L.) was occasionally added to stock cultures to retard growth of contaminating microorganisms, but was omitted from cultures during experiments.

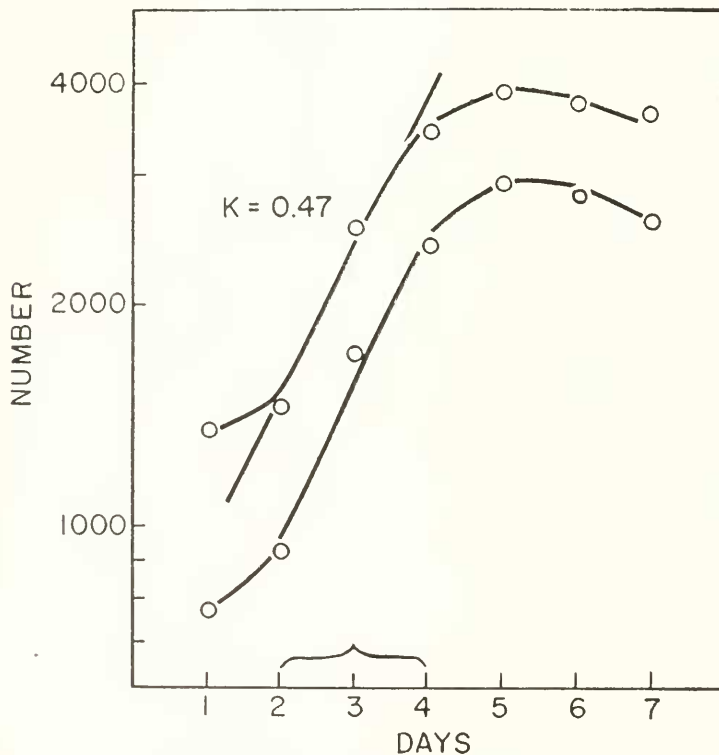


FIGURE 1. Growth curves for a stock culture of *C. viridissima* (green) obtained by counting hydranths (upper curve) or individual hydra (lower curve). Sampling period during logarithmic growth indicated by bracket.

Aposymbiotic control animals (referred to hereafter as albinos) were obtained by growing green individuals for eight days in culture solution containing 0.068 M glycerine (Whitney, 1907, 1908). Following this treatment, the absence of algae was confirmed by microscopic examination of macerated tissues and of 2-micron sections of paraffin-embedded whole animals. Aposymbiosis was permanent. Occasionally, algae reappeared in some individuals soon after glycerine treatment, apparently as a result of incomplete removal of algae. Under conditions defined elsewhere in this paper, albinos grew normally when returned to glycerine-free culture solution, and have continued to do so through three years

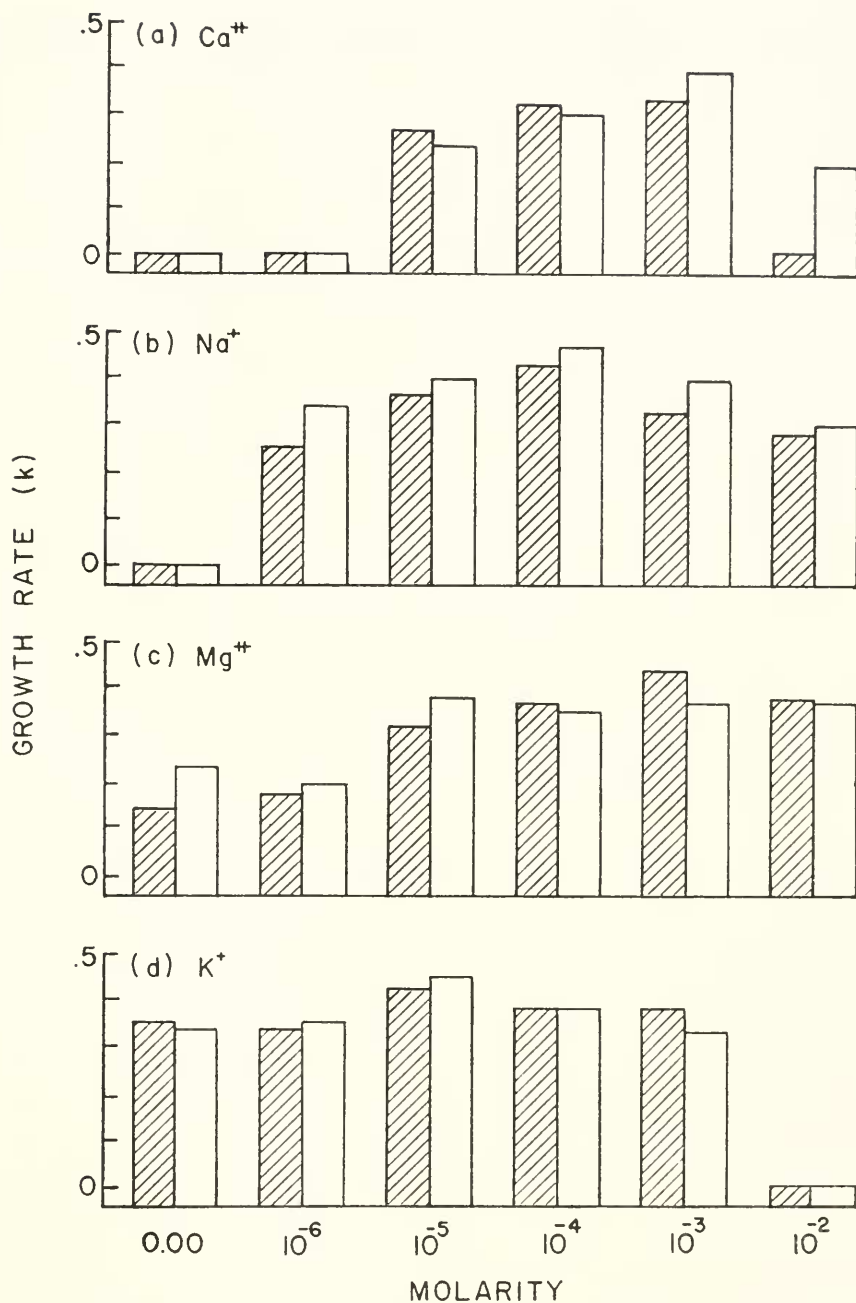


FIGURE 2. Histogram showing the growth rates of green (diagonal lined bars) and albino (open bars) *C. viridissima* in "M" solution in which the concentration of each of four cations is varied in turn while others held constant.

of subculture in our laboratory. Since the approximate life span of a *C. viridissima* cell is 2–3 weeks (Brien and Reniers-Decoen, 1949, 1950; Burnett and Garofalo, 1960), any undesirable effects of glycerine treatment would presumably be diluted or absent after a few months of subculture.

Stocks of hydra were cultured in Pyrex trays (33 cm. $\times$ 22 cm. $\times$ 4.5 cm.) and were fed daily on freshly hatched *Artemia* nauplii (cf. Loomis and Lenhoff, 1956). The hydra were kept at ambient laboratory temperatures (21–24° C.) and illumination; additional illumination was provided continuously from a 40-watt fluorescent light (Sylvania-Cool White) kept 15 cm. from the center of a stack of 2–4 trays. Stock cultures attaining a maximum of 4000 hydra/1500 ml. of culture solution were thinned and cleaned weekly by pooling the animals, washing them in deionized water and finally re-distributing 600–800 hydra (1200–1500 hydranths) to a tray containing 1500 ml. of clean culture solution. Throughout the course of these experiments, *C. viridissima* strain 1960 reproduced exclusively by asexual budding.

Hydra used in experiments were sampled from asexually-reproducing stock cultures during the logarithmic growth phase as illustrated in Figure 1. The maximum specific growth rate constant (k_{max}) for the stock population compares favorably with those values obtained for the growth rates of small experimental cultures (compare Fig. 1, Table II). Each sample consisted of duplicate groups of five hydra, each with a bud in an early stage of development, that were starved one day prior to the experiment. These animals can be defined as five "uniform" hydra (Lenhoff and Bovaird, 1961) or ten hydranths. Strict adherence to these sampling criteria was essential for reproducible experimental results.

Growth was measured by the procedure of Loomis (1954). Five uniform hydra were placed in 30 ml. of culture solution in a 10-cm. (diameter) Petri dish placed 10 cm. from a single 40-watt fluorescent light (Sylvania-Cool White).

TABLE I
Growth of green and albino C. viridissima in unmodified and modified culture solution

Culture solution	Number of hydranths on day						Growth rate k
	1	2	3	4	5	6	
A. "M" solution							
Green	10	15	25	32	50	91	0.433
Albino	10	16	26	38	53	87	0.433
B. "M" solution with 10^{-3} M NaCl instead of NaHCO_3							
Green	10	17	28	43	75	114	0.462
Albino	10	15	28	46	69	118	0.477
C. "M" solution plus 0.05 g./l. penicillin							
Green	10	15	23	40	60	105	0.477
Albino	10	15	23	38	60	100	0.462
D. "M" solution plus 0.068 M glycerine							
Green	10	15	19	30	38	64	0.355
Albino	10	14	18	27	43	57	0.346

TABLE II

Summary of growth rates of green and albino () C. viridissima measured prior to July, 1962. Culture conditions defined in text*

Group	No. of replicates	Mean k	Range
1	2	0.46	0.00
2	6	0.45	0.03
3	2, 2*	0.45	0.06
4	2, 2*	0.43	0.05
5	3	0.46	0.11
6	2	0.49	0.00
7	2	0.55	0.04
8	2	0.45	0.08
9	2	0.43	0.05
10	2	0.42	0.02
11	3	0.43	0.04
12	2*	0.45	0.04
13	2	0.42	0.01
14	2	0.45	0.05
15	2*	0.39	0.00
16	2	0.31	0.06
17	2	0.37	0.01
18	1, 2*	0.39	0.04
19	3	0.32	0.02
20	2	0.32	0.02
21	2*	0.36	0.01
Mean values			
entire group	(n = 54)	0.42 ± 0.06	0.29
green only	(n = 42)	0.42 ± 0.06	0.29
albino only	(n = 12)	0.41 ± 0.03	0.12

The number of hydranths was counted daily and then a dense suspension of *Artemia* nauplii was introduced to each hydranth in the dish with a glass pipette. Hydranths which happened not to catch larvae within the first few minutes were given *Artemia* with watchmaker's forceps. The culture medium was renewed one hour after feeding and again six hours later after the normal process of regurgitation was completed. This procedure was followed for 5-7 days. A semi-logarithmic plot of the number of hydranths present on successive days (*cf.* Table Ia) yielded a series of points. From a straight line fitted to these points, k , the growth rate constant, was calculated using the standard equations for logarithmic growth. These can be expressed finally as $k = \ln 2/T$, where T is the doubling time in days, estimated to the nearest tenth of a day (*cf.* Loomis, 1954).

RESULTS

Environmental cations required for growth

Figure 2 shows the results of a series of growth experiments in which the concentration of one environmental cation was varied while that of the others was kept constant as in "M" solution. The effect of the varied cation on growth of

green and albino *C. viridissima* was determined from the value for k . Growth at or below a rate characteristic of starved hydra was assigned a k value of 0.00.

C. viridissima required at least 10^{-5} M calcium (Fig. 2a) and 10^{-6} M sodium (Fig. 2b) ions for growth. In the absence of these ions the animals did not grow, failed to feed, remained contracted and were prone to disintegration within a few days.

In the absence of magnesium ions, budding rates were usually low (Fig. 2c), approaching those of starved animals, but occasionally maximum growth was observed. Addition of at least 10^{-5} M magnesium ions always enhanced growth rates.

Although growth occurred without potassium (Fig. 2d) its presence in the medium markedly enhanced the general appearance of the polyps and the reproducibility of growth curves. Average length of tentacles increased about three-fold when potassium was present.

High concentration of calcium and potassium inhibited growth (Fig. 2a, d), while similar high concentrations of sodium or magnesium were without effect (Fig. 2b, c).

The general growth response of green and albino *C. viridissima* to all ions tested was nearly the same with two exceptions: (1) albinos appeared more sensitive to lack of environmental sodium, disintegrating several days earlier than green hydra, and (2) green hydra produced significantly fewer buds than did albinos at high calcium concentrations.

Anions and other factors

Growth increased slightly but not significantly when NaCl was substituted for NaHCO_3 (Table Ib), but HCO_3^- was retained in all subsequent experiments to insure an ample external carbon dioxide pool for the algae.

When penicillin was added to stock cultures (Table Ic), the growth rate increased slightly. Glycerine (0.068 M) decreased the growth rate considerably (Table Id), and was used only to eliminate symbiotic algae.

Growth rate under standard conditions

Table II summarizes the growth rates of *C. viridissima* measured in our laboratory prior to July, 1962. Standard conditions are defined as growth in laboratory culture solution, pH 7.6, 21–24° C., continuous illumination (see Methods), daily feeding on freshly hatched live *Artemia* nauplii, twice-daily changes of the culture solution, constant volume of culture solution per vessel and initiation of growth experiments with uniformly developed hydra of known nutritional history sampled during the logarithmic growth phase of stock populations. The 54 measurements include data for 42 groups of green and 12 of albino *C. viridissima*. Under these conditions, albinos grow at about the same logarithmic rate as green individuals.

Using an analysis of hydroid growth measurements as set forth by Fulton (1962), the standard deviation of the mean growth rate of the whole group (54 measurements) is ± 0.06 ; the range, 0.29. For each individual set of experiments (21 measurements) the standard deviation of the mean is ± 0.03 (calculated from

ranges), the range, 0.11. Thus, the variation encountered from one group of experiments to another is about twice that encountered among replicates within any single experiment, indicating that conditions within each set of experiments were relatively constant.

In 20 of 21 experiments, the growth rate of replicates differed from each other by 0.08 or less. In one experiment a difference of 0.11 was observed. From these data we may estimate that 95% of the time a growth rate difference of 0.08 or more between cultures is significant.

DISCUSSION

The results demonstrate that in a solution containing calcium, sodium, magnesium, potassium, chloride, and bicarbonate in the concentrations described, growth of *C. viridissima*, Carolina strain 1960, is exponential with a mean k of 0.42 ± 0.06 . This represents a doubling time of 1.45–1.90 days. This is the

TABLE III

*Comparison of ionic requirements for growth of several hydroids in laboratory culture.
Table modified from Fulton (1962)*

	<i>H. littoralis</i> *	<i>C. lacustris</i>	<i>C. viridissima</i>
Growth curve	exponential	exponential	exponential
k	0.37	0.21	0.42
Cations required	Ca ⁺⁺ (10^{-5} M) Na ⁺ (10^{-6} M)**	Ca ⁺⁺ (10^{-3} M) Na ⁺ (10^{-3} M) K ⁺ (10^{-3} M)	Ca ⁺⁺ (10^{-5} M) Na ⁺⁺ (10^{-6} M)
Cations which enhance growth but are not required	K ⁺⁺⁺	Mg ⁺⁺	K ⁺ , Mg ⁺⁺

* Data of Loomis (1954).

** Data of Lenhoff and Boyaird (1960).

*** Data of Lenhoff (unpub.).

fastest mean growth rate thus far encountered among laboratory-grown hydroids for which there are comparable data (see Table III) and in part reflects the smaller size of *C. viridissima*. The observation that the mean growth rates of green and albino *C. viridissima* are nearly identical *under the conditions described* suggests that the algae are not essential for growth at $k_{\max}$. This observation is dealt with in the next paper in this series.

Using data of Loomis (1954) and Fulton (1962) some growth features of *Hydra littoralis* (a stream-dwelling non-symbiotic species, about 3–4 times larger than *C. viridissima*) and *Cordylophora lacustris* (a colonial hydroid typically found in fresh to brackish situations) are compared with those of *C. viridissima* in Table III. All have been grown in the laboratory under controlled conditions. All three hydroids require calcium ions in the environment for growth, although the minimal required concentrations vary with species. In the absence of calcium, the animals disintegrate very quickly, reflecting the fundamental role of this ion in maintaining cell and tissue integrity. At low levels of calcium, other factors

which may adversely affect growth are (1) loss of calcium-dependent contractility necessary for positioning body and tentacles in prey capture and feeding; (2) failure of nematocysts to discharge even when contacted by live *Artemia* nauplii; (3) inability to carry out the feeding reflex for which environmental calcium is required (Lenhoff and Bovaird, 1959). The animals are thus effectively prevented from feeding and obtaining additional calcium from food.

C. viridissima also shares with *Hydra littoralis* and *Cordylophora* an absolute requirement for sodium in the external environment. Only trace amounts of sodium appear necessary for near-optimum growth of the fresh-water hydroids (*H. littoralis* and *C. viridissima*), while *Cordylophora*, as expected from its habitat, tolerates a relatively higher environmental sodium concentration.

Environmental potassium ions are not an absolute requirement for growth of *C. viridissima* or *Hydra littoralis*, but when present, even at low concentration (10^{-6} M) enhance the general appearance of polyps and the level of reproducibility of growth rates. The dramatic increase in length of tentacles observed when potassium is introduced into the medium may reflect the role of this cation in nerve and muscle irritability. Since tentacle length:body length ratio is often used as a taxonomic character in identification of hydras (Hyman, 1929), standardized culture media may help eliminate uncertainty in this parameter.

Although environmental magnesium definitely enhances growth of *C. viridissima* (cf. Muscatine, 1961), it is uncertain as to whether or not it is absolutely required since maximum growth was occasionally observed in the absence of this cation. Magnesium also appears to be "less critical" for *Cordylophora* (Fulton, 1962). Acquisition of magnesium from food may offset a deficiency in the medium and thus interfere with the reproducibility of the effects of absence of environmental magnesium on growth. A magnesium requirement for *C. viridissima* is apparently not primarily related to a requirement by the algae since the growth rate of albinos is also lower in the absence of magnesium.

Inhibition of growth of *C. viridissima* at high concentrations (10^{-2} M) of calcium and potassium was probably not an osmotic effect, but more likely related to competition with other ions since similar osmolar concentrations of sodium and magnesium did not inhibit growth. Sodium-potassium competition is a well-known phenomenon, and has been demonstrated in hydroids by Fulton (1962) in growth experiments using *Cordylophora*.

There is evidence that symbiotic algae may affect mechanisms of salt and water balance of the host. For example, in these studies albino *C. viridissima* disintegrated several days sooner than green *C. viridissima* in the absence of sodium. The same effect was observed in a medium also lacking in magnesium (Lenhoff and Bovaird, 1960). On the other hand, albinos were less sensitive to high calcium compared to green individuals. Karakashian (1963) noted that algae-free *Paramecium bursaria* crenated and died within a few hours after inoculation into an inorganic salt medium in which normal green individuals could be maintained. Hood (1927) observed that specimens of *Frontonia leucas*, another ciliate harboring symbiotic algae, lived indefinitely in a 1% dextrose solution changed daily, and for several days when adapted to 3% dextrose. In contrast, algae-free individuals averaged two days' survival in 1% dextrose and died within a few hours in more concentrated solutions. However, in distilled water

green specimens disintegrated immediately while those without symbionts survived for several hours.

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SUMMARY

1. Under controlled conditions in the laboratory, with daily feeding, *Chlorohydra viridissima* grew exponentially in a solution containing calcium, sodium, magnesium, potassium, chloride and bicarbonate ions. The optimum concentration of cations was ascertained and the effect of their deficiencies noted. The results are compared with data for *Hydra littoralis* and *Cordylophora lacustris*.

2. Calcium and sodium ions were required for growth. Magnesium and potassium enhanced growth and reproducibility of growth rates. Bicarbonate was not essential.

3. Both symbiotic and aposymbiotic *C. viridissima* grew at nearly identical rates, doubling in number every 1.45–1.90 days. In the absence of environmental sodium aposymbiotic hydra disintegrated several days sooner than normal green individuals, while growth of the latter was inhibited by high concentrations of calcium in the environment.

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